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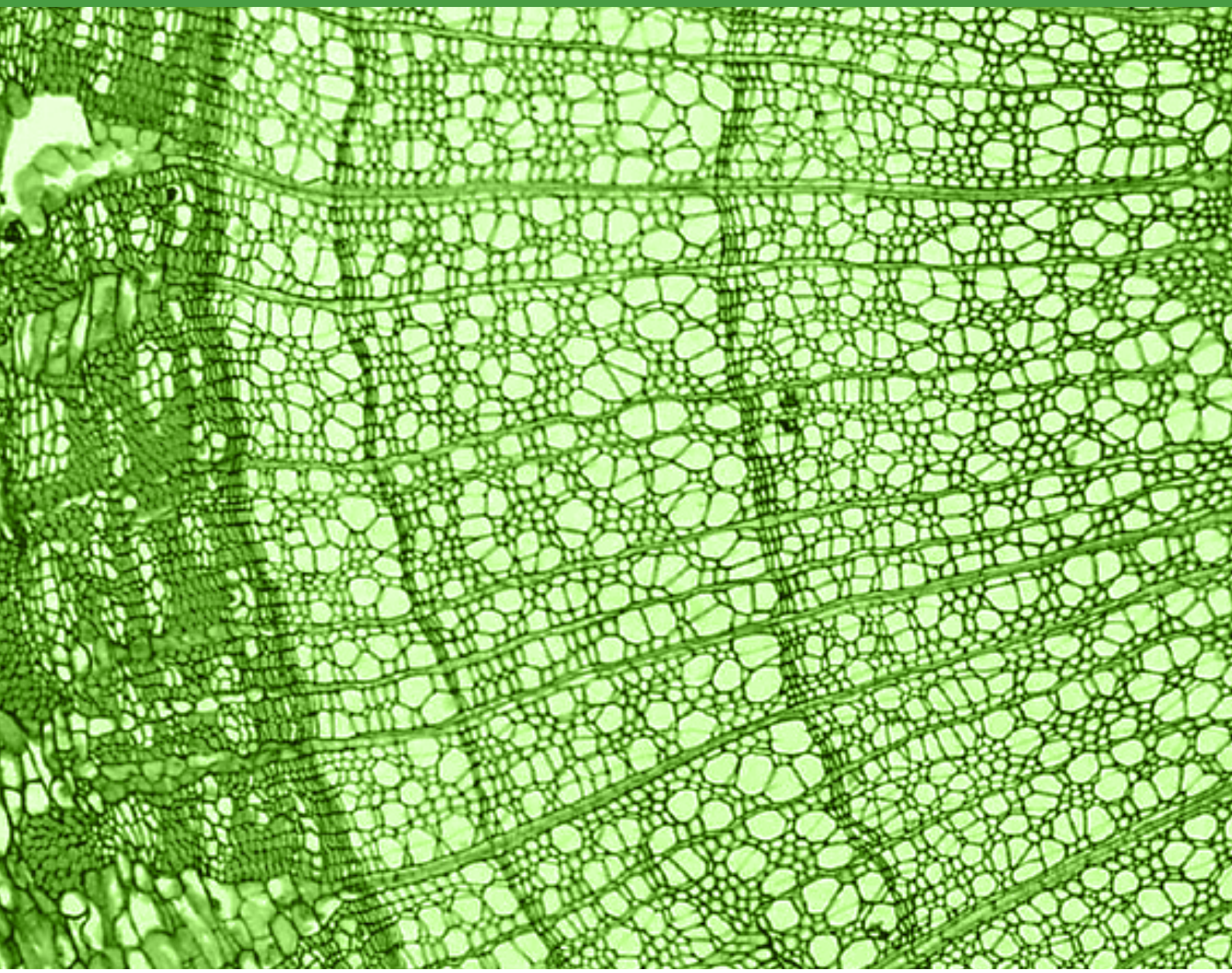

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PHYSICAL AND FUNCTIONAL INTERACTION OF CARBONIC ANHYDRASES AND NBCe1 Na^+/HCO_3 COTRANSPORTER IN THE HEART. THE METABOLON REVISITED.

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Running title: NBCe1/CA complex in the myocardium

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Abstract

To allow the control of their intracellular pH (pH_i) and bicarbonate (HCO_3^-) levels, cells express HCO_3^- transport proteins (NBC) that rapidly and selectively move HCO_3^- across the plasma membrane. In the heart electroneutral NBCn1 and electrogenic NBCe1 Na^+/HCO_3^- cotransporters facilitate the transmembrane movement of HCO_3^- ions into cardiomyocytes, as a response to acid loading. NBCe1 associates with carbonic anhydrases (CA), the enzymes that catalyze the reversible conversion of CO_2 to HCO_3^-, to form a transport metabolon, a weakly associated complex of sequential metabolic enzymes. NBCe1 physically/functionally interact with the isoforms II, IV, and IX of CA, to increase the HCO_3^- flux through cell membranes. NBCe1 and CAs interaction occurs in different cellular compartments in the heart muscle. Physiologically, the NBCe1/CA complex could contribute to the removal of H^+ ions accumulated as the result of the contractile activity of the cardiac muscle cell, and this process may occur at the surface sarcolemma (CAII-NBCe1-CAIV complex) or at the t-tubule (CAII-NBCe1-CAIX complex) of the cardiomyocyte. Pathologically, up-regulation of the NBCe1/CA metabolon system upon ischemic/hypoxic conditions of the heart would favor the hypertrophic growth of the cardiac cells.

Keywords: NBC/CA complex; Metabolon; Heart; Bicarbonate

Introduction

Intracellular pH (pH_i) is an important modulator of the excitation and contraction process in the heart. Two major transporters are responsible for acid extrusion in cardiomyocytes, the Na^+/H^+ exchanger (NHE1) and the Na^+/HCO_3^- co-transporter (NBC), which transports H^+ out and HCO_3^- into the cell, respectively [1,2].

Different NBC isoforms catalyzed the Na^+-coupled HCO_3^- movement in the heart, the electrogenics NBCe1 (NBC1) [3,4] and NBCe2 (NBC4) [5,6], encoded by the *SLC4A4* gene [4], and the *SLC4A5* gene [5,7], respectively, and the electroneutral NBCn1 (NBC3), encoded by the *SLC4A7* gene [8]. In recent studies, however, we only identified functional NBCe1-dependent electrogenic Na^+/HCO_3^- cotransport movement in isolated cardiomyocytes [9], which questioned the functional role of NBCe2 in the mammalian heart, as previously suggested [10].

Carbonic anhydrases (CA) catalyze the reversible hydration of CO_2, contributing to HCO_3^- and H^+ formation. Sixteen members of the CA family differing in their catalytic activities, tissue distribution and subcellular localization have been characterized in mammalian cells [11]. CAIV, CAIX, and CAXIV are expressed in different sub-cellular compartments within heart cells [12], suggesting divergent functions of the enzymes in relation to the contractile function of the cardiomyocytes. Besides the expression of mitochondrial CAV [13] and intracellular CAII [14], cardiac cells express membrane-anchored CAIV, and transmembrane CAIX and CAXIV [12].

Physical NBC and CA interaction

Several evidences have demonstrated that CA enzymes and HCO_3^- transporter (BT) proteins are functionally coupled; CA alternatively produces or consumes HCO_3^-, the substrate for these transporters. Previously, the cytosolic CAII enzyme was found to form a complex with the kidney variant of NBCe1 (kNBC1), functioning as a HCO_3^- transport metabolon (BTM) [15,16]. Moreover, coexpression of CAI, or CAII, or CAIII and NBCe1 worked as a functional complex in the *Xenopus laevis* oocyte system [17,18]. Conversely, *Lu and collaborators* did not find any functional implication when CAII and NBCe1 were co-expressed in the same system [19]. In addition, physical interactions between NBCe1 (NBC1b) and CAIV was demonstrated at the plasma membrane in renal tissues [16]. This interaction is required for full NBCe1 activity [16]. We have recently explored the interaction of the NBCe1 with different CAs in cardiac tissues [20]. NBCe1 was immunoprecipitated by anti-CAII and anti-CAIV antibodies, revealing association of NBCe1 with cytosolic and glycosyl-phosphatidyl-inositol (GPI)-anchored CA enzymes, respectively (Fig. 1A).

Transmembrane CAIX is localized almost exclusively to the t-tubular region of cardiac muscle [12], and skeletal muscle cells [21]. We have recently localized NBCe1 in t-tubules and sarcolemmal membranes of isolated cardiomyocytes [9]. In agreement to our study, *Garciarena et al* [22] have recently confirmed this localization of NBC in the heart. The similar cardiomyocyte localization pattern and previously reported interaction of NBCe1 and CAs into BTM [16], suggest that NBCe1 might functionally and structurally associate with CAIX in the heart. Consistently, we found that NBCe1 could be coimmunoprecipitated using anti-CAIX antibody in the rat heart (Fig. 1A) and in the HEK293 cells heterologous expression system (Fig. 1B) [20]. In addition, using immunocytochemistry combined with confocal microscopy, we demonstrated that CAIX and NBCe1 colocalized in cardiomyocyte compartments reminiscent of t-tubules

but not at the surface sarcolemma [20]. These experiments suggest that NBCe1 and CAs present in different cellular compartments interact in the heart muscle.

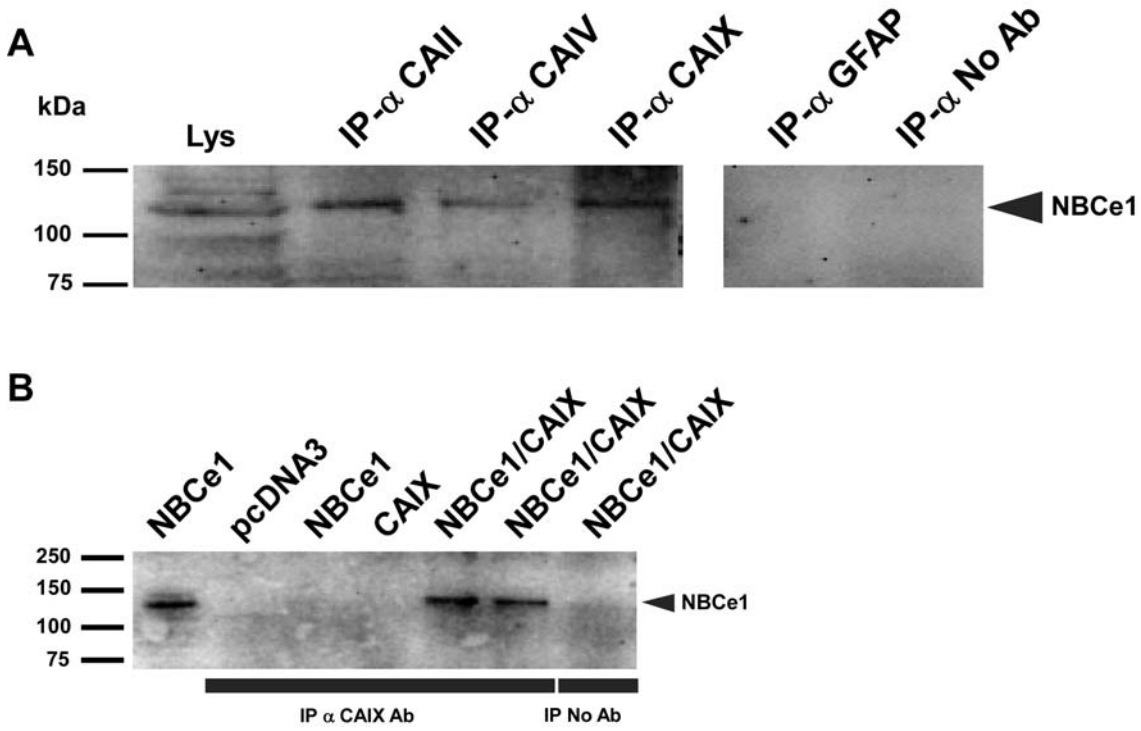

Figure 1. Coimmunoprecipitation of carbonic anhydrases and NBCe1 from heart and HEK293 cells. A, Adult rat ventricular lysates were immunoprecipitated (IP) with antibody directed against CAII, CAIV, CAIX, irrelevant glial fibrilar acid protein (GFAP), or without antibody (No Ab). Samples were resolved by SDS-PAGE on 8% gels, blotted, and probed with an anti-NBCe1 antibody (arrow). **B**, HEK293 cells were individually transiently transfected with NBCe1, CAIX, or pcDNA3 (empty vector), cDNA, or cotransfected with NBCe1 and CAIX, cDNAs. Cell lysates were immunoprecipitated (IP) with anti-CAIX antibody (M75), or IP without antibody (no Ab), resolved by SDS-PAGE, blotted, and probed with an anti-NBCe1.

Molecular site of NBCe1 and CAIX interaction

Without the help of crystal structures, the identification of particular NBCe1 protein regions exposed to the extracellular milieu with the only assist of molecular and biochemical approaches is not definitive. On the basis of previous topology studies of the human AE1 Cl^-/HCO_3^- exchanger [23], which is ~30% identical to NBCe1, extracellular loops 3 (EC3) and 4 (EC4) of NBCe1, could be assumed. The NBCe1-EC4 region was previously identified as responsible for the NBCe1/CAIV interaction [16]. Recently, *Chen et al* confirmed that the EC4 of NBCe1 is exposed to the extracellular fluid, directly or indirectly controlling the functional nature of the transporter [24]. Accordingly, we found that the extracellular EC4 region of NBCe1 binds CAIX. A role of the large ECs in NBCe1 function, in substrate funnelling to the transport site, was suggested by the result that antibodies against EC3 or EC4 of NBCe1 were able to interfere with NBCe1 transport activity [9]. While antibodies produced against EC3 blocked NBCe1 activity in a similar fashion to the stilbene-derivatives [3,25], antibodies raised against a peptide corresponding to putative EC4 of NBCe1 produced an stimulatory effect on NBCe1 activity [9]. Similar function-blocking effects of

antibodies directed against putative EC3 of the AE3 BT, has been observed [26]. Functional implications of the putative large EC of NBCn1 and NBCe1 have been recently described [24]. EC4 of NBCe1, which is exposed to the extracellular fluid, was suggested to either contribute to the substrate-binding vestibule or indirectly influence substrate binding by interacting with different transmembrane segments, controlling the nature of the transport by NBCe1 [24].

Functional NBC and CA interaction

As already mentioned, NBC are crucial in the regulation of pH_i and HCO_3^- metabolism. Electrogenic NBCe1 catalyzes HCO_3^- fluxes in heart cells. Cytosolic CAII associate with NBCe1 at the inner surface of the plasma membrane, and the NBC1/CAII interaction is needed for full NBCe1-mediated pH_i recovery activity after cellular acid loading [16]. In addition, the tethering of CAIV close to the NBCe1 HCO_3^- transport site, maximizes the transmembrane HCO_3^- gradient local to NBCe1 and thereby activates the transport rate [16].

We have also recently analyzed the NBCe1/CAIX functional coupling by examining the effect on NBCe1 transport activity of CA inhibitors; the membrane-permeable 6-ethoxyzolamide (ETZ) or the poor membrane-permeable benzolamide (BZ) (Fig. 2) [20]. We examined NBCe1 transport activity by subjecting isolated cardiomyocytes, loaded with a fluorescent pH indicator, to membrane potential depolarizing pulses, which selectively activates the electrogenic NBC. Both ETZ and BZ CA inhibitors partially inhibited the hyperkalemic-induced NBCe1-dependent depolarization and consequent intracellular alkalinization, in isolated rat ventricular myocytes (Fig. 2A) [20]. Membrane-permeable ETZ inhibited NBCe1 activity by 65% compared to control, at the maximal alkalinization point registered (15 min) (Fig. 2B). Conversely, BZ inhibited the maximal NBCe1-mediated alkalinization by 35% compared to control (Fig. 2B).

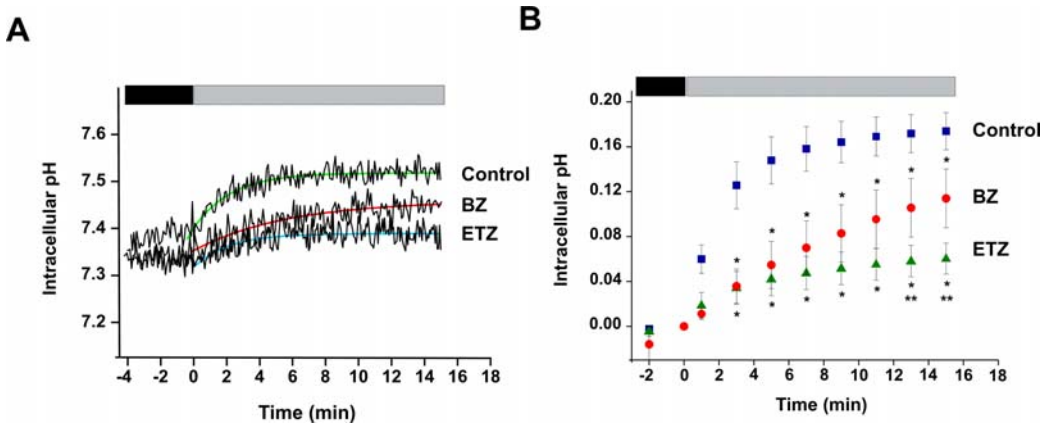

Figure 2. E**ffect of carbonic anhydrase inhibition on NBCe1 activity A**, Representative trace of pH_i recorded from an isolated rat cardiomyocyte exposed to a K^+ pulse in control and in the presence of the CA inhibitors, benzolamide (BZ, 100 μM), or 6-ethoxyzolamide (ETZ, 100 μM). Black bar= 5 mM K^+_o; Grey bar= 45 mM K^+_o. **B**, Average data of pH_i alkalinization produced by the hyperkalemia-induced depolarization of membrane potential in control (n=11, blue) and in the presence of the CA inhibitor, BZ (100 μM, n=7, red) or the CA inhibitor, ETZ (100 μM, n=6, green). Data are expressed as increase of pH_i units in comparison to the zero time point in high K^+. * indicates $P<0.05$ vs. control, and ** indicate $P<0.05$ vs. BZ

The results indicate that the activity of NBCe1 is maximized by CA. Differences in the inhibitory profile achieved by ETZ and BZ on NBCe1 activity could be explained by selective inhibition of intracellular (CAII) and extracellular (CAIV, CAIX, CAXIV) NBCe1-bound CAs, in rat cardiomyocytes.

Physiological relevance of the NBC/CA Metabolon in myocardium

As commented above, CAIV and CAIX seem to be coupled to NBCe1 at the sarcolemmal and t-tubules, respectively (Fig. 3). Coupling of CAIX and NBCe1 in the t-tubules of cardiomyocytes may serve to maximize the acid secretory capacity of the cell, and participate in the movement of a CO_2/HCO_3^- diffusional shuttle from the lumen of the tubule toward the interstitial space. The constant accumulation of H^+ ions as the result of the contractile activity of the cardiac muscle cell may be optimally dissipated by the NBCe1/CAIX complex from the t-tubule environment (Fig. 3). In addition, the NBCe1/CAIV metabolon also contribute to the H^+ ions removal, an association that take place most likely at the sarcolemma of cardiomyocyte. A similar effective pathway of acid removal have been proposed for the CA-facilitated lactic acid transport in fast mouse extensor skeletal muscles, whereby about half of the CA-dependent muscular lactate flux occurs across the surface membrane, while the other half occurs through t-tubuli membranes [21]. Yet, on the basis of our functional data analyzed in the physiological context (cardiomyocytes) and with the use of widely characterized CA inhibitors, we found that CAs bound to NBCe1 enhance electrically-coupled movement of HCO_3^- ions by the NBCe1 cotransporter. We propose that CAIX form a tight functional complex with NBCe1, and that the mechanism by which transmembrane CAIX regulate pH_i in cardiomyocytes occurs through the efficient uptake of HCO_3^- locally formed in the ‘‘mouth’’ of the NBCe1 by the CA. In addition, other BT expressed in cardiac muscle would bind to CAIX, forming other BTM and contributing to pH_i regulation in the heart.

This model proposes a local change of HCO_3^- gradient driven by the catalytic activity of extracellular CAIX created in a microenvironment such as the t-tubule and transported via the NBCe1 BT (Fig. 3).

The rate of transport by NBCe1 in cardiomyocytes is proportional to the concentration gradient for HCO_3^- across the membrane; transport is maximized by high local concentration of HCO_3^- at the *cis* side (extracellular) of the membrane provided by the binding of CAIX/CAIV, and low HCO_3^- at the *trans* side (cytoplasmic), which is dissipated by the binding of CAII (Fig. 3). The combined effects of CAII and CAIX/CAIV provide a *push-pull* effect on transport.

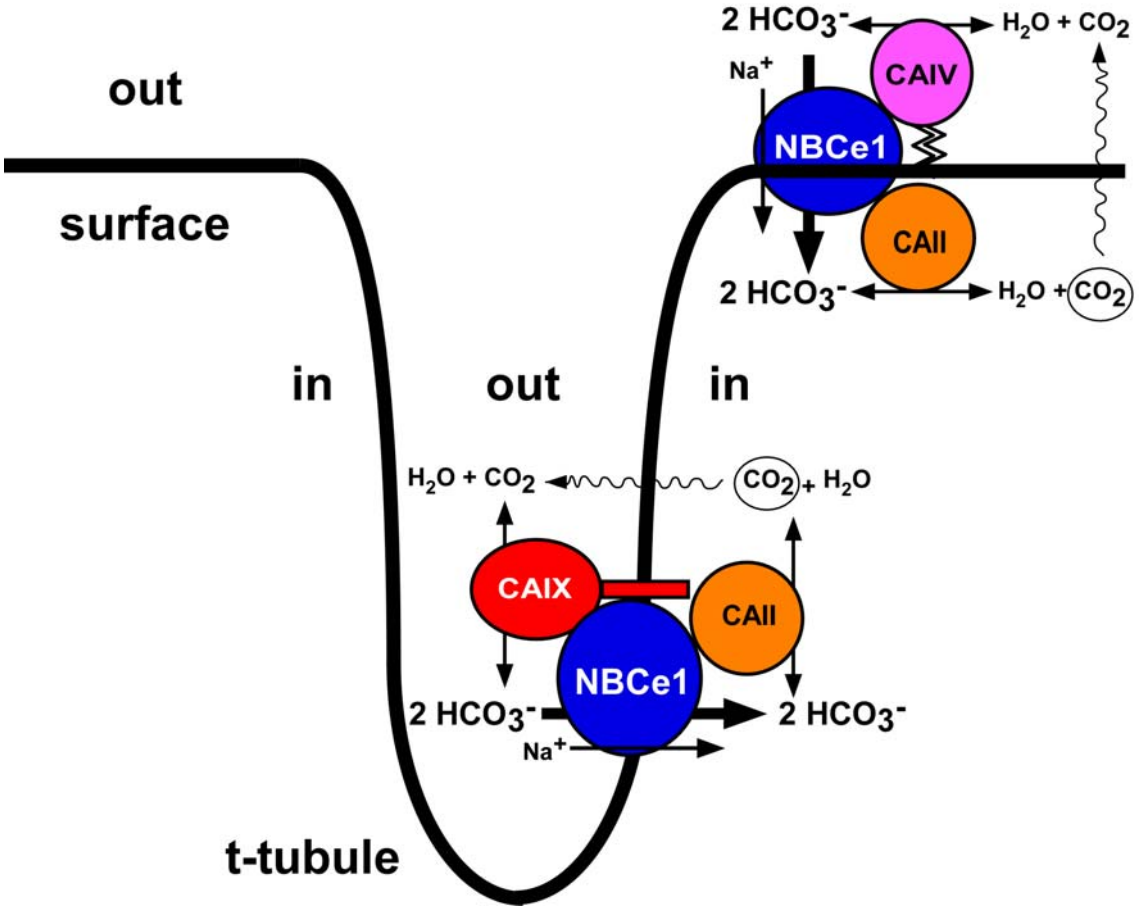

Figure 3. NBCe1 bicarbonate transport indicating the push-pull effect of CAIX, CAII and CAIV in cardiomyocytes. Schematic model of the binding of the transmembrane CAIX, GPI-anchored CAIV, and cytoplasmic CAII to NBCe1 protein, in the surface and t-tubule membrane of cardiomyocytes. The multi-molecular arrangement potentiates NBCe1-mediated HCO_3^- flux by the production and removal of HCO_3^- from the transport site. CAIX and CAII in the t-tubule and CAIV and CAII in the surface membrane of the cell cooperate to push bicarbonate to the transport site and then pull bicarbonate from the opposite side of the membrane. NBCe1 operates with a 1 Na^+:2 HCO_3^- stoichiometry in the heart, as visualized in the figure. The structure attached to the CAIV represents the GPI anchor that couples CAIV to the lipid bilayer, and the structure protruding from the CAIX extracellular catalytic domain represents the transmembrane and cytoplasmic domains of the protein. Contorted line shows free diffusive movement of acid in the form of CO_2, from the intracellular (in) to the extracellular (out) compartments of the cell.

Potential pathological relevance of the NBC/CA Metabolon in myocardium

Changes of NBC expression have been associated with cardiac pathology. Levels of NBCe1 mRNA increased twofold in the rat left ventricle after myocardial infarction [27]. Also, in human ischemic and dilated cardiomyopathic heart which is commonly accompanied by hypoxia, NBCe1 mRNA expression increased whereas mRNA level of NBCn1 remained unchanged [28]. Conversely, in a rat model of pressure overload cardiac hypertrophy, hypertrophied myocytes showed a marked increased expression of NBCe1 and NBCn1 [10]. In both non-ischemic [10], or ischemic hypertrophic hearts [27,28] increased NBCe1 expression was accompanied by

enhanced NBCe1-mediated HCO_3^- fluxes. Up-regulation of NBCe1 may have significant physiological consequences for hypertrophied myocytes. For instance, it may promote arrhythmias and reperfusion injury as a result of its ability to cause $[Na^+]_i$ accumulation leading to $[Ca^{2+}]_i$ overload via sarcolemmal Na^+/Ca^{2+} exchange. Inhibition of NBCe1 has been shown to reduce myocardial damage in normal rat ventricle subjected to ischemia [28]. Furthermore, normal ventricular myocytes display a significant rise in $[Na^+]_i$ when NBC is activated by intracellular acidosis [29]. Because $[Na^+]_i$ overload promotes hypertrophic development [30,31], it also seems possible that chronic attenuation of Na^+ influx via NBC may reduce remodeling as observed by NHE1 inhibition in rats subjected to myocardial infarction [32].

CAII and CAIV mRNA and protein levels rose in cultured neonatal and adult cardiomyocytes subjected to the hypertrophic phenylephrine (PE) and angiotensin II (AngII) treatment, supporting the notion that CAII/CAIV activation is a component of the hypertrophic pathway [14]. Interestingly, the CA inhibitor ETZ both prevented and reversed PE-induced hypertrophy. In addition, ETZ and a related CA inhibitor methazolamide prevented hypertrophy in adult cardiomyocytes exposed to AngII [14].

CAIX gene is under control of the hypoxia-inducible factor 1 (HIF-1) transcription factor and is associated with tumor cell growth and poor survival, in patients with cancer [33,34]. In addition, CAIX promoted tumor growth and conferred survival advantage to cells exposed to hypoxic and acidic microenvironments [35]. On the other hand, the contractile function of the heart is compromised under low O_2 content, and the lack of O_2 might be challenging for an organ that works under aerobic conditions. In this line, cultured cardiomyocytes and adult rats exposed to hypoxic conditions increased CAIX expression compared to cells or rats maintained in normoxia [36]. Also, chronic hypoxic conditions induced increase in the NBC expression in the human skeletal muscle [37].

According to the pathological conditions described above, up-regulation of the NBCe1/CAIX metabolon upon pathological ischemic/hypoxic conditions would favor the hypertrophic growth of the heart.

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